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PACKAGING CELL LINES

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/100,063, filed September 12, 1998 and U.S. Provisional Application No. 60/100,022, filed September 11, 1998, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Retroviral vectors based on lentiviruses, such as human immunodeficiency viruses (HIV), can infect nondividing cells, and integration of proviral DNA occurs without the need for cell division. These properties make lentiviruses attractive for gene transfer into nondividing cells, such as hepatocytes, myofibers, hematopoietic stem cells, and neurons.

However, the use of lentivirus vectors, particularly HIV vectors, particularly for gene therapy, is hampered by concern over their safety. Thus, a need for the development of lentivirus vectors, particularly HIV vectors, with improved safety, particularly for gene therapy, exists.

SUMMARY OF THE INVENTION

The present invention relates to novel packaging cell lines useful for generating viral accessory protein independent lentivirus-derived, particularly HIV-derived,

retroviral vector particles, to construction of such cell lines and to methods of using the accessory protein independent lentivirus-derived retroviral vector particles to introduce DNA of interest into cells (e.g, eukaryotic cells such as animal (particularly mammalian), plant or yeast cells or prokaryotic cells such as bacterial cells). In a preferred embodiment, the packaging cell lines of the present invention are stable packaging cell lines.

In one embodiment of the invention, packaging cell lines for producing a viral accessory protein independent lentivirus-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); and (b) a retroviral nucleotide sequence in the cell which comprises a coding sequence for lentivirus *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the lentivirus gagpol proteins.

In second embodiment of the invention, packaging cell lines for producing a viral accessory protein independent lentivirus-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); (b) a first retroviral nucleotide sequence in the cell which comprises a coding sequence for lentivirus *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the lentivirus gagpol proteins; and (c) a second retroviral nucleotide sequence in the cell which comprises the coding sequence for a heterologous envelope protein.

In a third embodiment of the invention, packaging cell lines for producing a viral accessory protein independent lentivirus-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); (b) a first retroviral nucleotide sequence in the cell which comprises a coding sequence for lentivirus *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the lentivirus gagpol proteins; (c) a second retroviral nucleotide sequence in the cell which comprises the coding sequence for a heterologous envelope protein; and (d) a third retroviral nucleotide sequence which comprises a DNA sequence of interest and lentivirus cis-acting sequences required for packaging, reverse transcription and integration.

In a fourth embodiment of the invention, packaging cell lines for producing a viral accessory protein independent lentivirus-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); (b) a retroviral nucleotide sequence in the cell which comprises a coding sequence for lentivirus *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the lentivirus gagpol proteins; and (c) a retroviral nucleotide sequence which comprises a DNA sequence of interest and lentivirus cis-acting sequences required for packaging, reverse transcription and integration.

In a fifth embodiment of the invention, packaging cell lines for producing a viral accessory protein independent HIV-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); and (b) a retroviral nucleotide sequence in the cell which comprises a coding sequence for HIV *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the HIV gagpol proteins.

In sixth embodiment of the invention, packaging cell lines for producing a viral accessory protein independent HIV-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); (b) a first retroviral nucleotide sequence in the cell which comprises a coding sequence for HIV *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the HIV gagpol proteins; and (c) a second retroviral nucleotide sequence in the cell which comprises the coding sequence for a heterologous envelope protein.

In a seventh embodiment of the invention, packaging cell lines for producing a viral accessory protein independent HIV-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); (b) a first retroviral nucleotide sequence in the cell which comprises a coding sequence for HIV *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the HIV gagpol proteins; (c) a second retroviral nucleotide sequence in the cell which comprises the coding sequence for a heterologous envelope protein; and (d) a third retroviral nucleotide sequence which

comprises a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration.

In a eighth embodiment of the invention, packaging cell lines for producing a viral accessory protein independent HIV-derived retroviral vector particles comprise (a) a cell (e.g., mammalian cell); (b) a retroviral nucleotide sequence in the cell which comprises a coding sequence for HIV *gagpol*, wherein said coding sequence has been codon optimized by mutagenisis to improve expression of the HIV gagpol proteins; and (c) a retroviral nucleotide sequence which comprises a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration.

Alternatively, each of the packaging cell lines described herein can be produced using (1) a retroviral nucleotide sequence which comprises a codon optimized gag coding sequence and (2) a retroviral nucleotide sequence which comprises a codon optimized pol coding sequence, in place of the retroviral nucleotide sequence which comprises a codon optimized gagpol coding sequence.

In a particular embodiment, the heterologous envelope protein is the G glycoprotein of vesicular stomatitis virus (VSV G). In another embodiment, the heterologous envelope protein is the amphotropic envelope of the Moloney leukemia virus (MLV).

Cell lines for producing a viral accessory protein independent lentivirus-derived retroviral vector particles are produced by transfecting host cells (e.g., mammalian host cells) with a plasmid comprising a DNA sequence which encodes lentivirus *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis to improve expression of the lentivirus gagpol proteins. Depending upon the particular cell line being produced, the host cells are also co-transfected with a plasmid comprising a DNA sequence which encodes a heterologous envelope protein, or a plasmid comprising a DNA sequence of interest and lentivirus cis-acting sequences required for packaging, reverse transcription and integration, or both of these plasmids. Alternatively, host cells are transfected with a plasmid comprising a codon optimized

DNA sequence encoding a lentivirus gag protein and a plasmid comprising a codon optimized DNA sequence encoding a lentivirus pol protein, in place of the plasmid comprising a codon optimized DNA sequence encoding both lentivirus gagpol proteins.

Cell lines for producing a viral accessory protein independent HIV-derived retroviral vector particles are produced by co-transfecting host cells (e.g., mammalian host cells) with a plasmid comprising a DNA sequence which encodes HIV *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis to improve expression of the HIV gagpol proteins. Depending upon the particular cell line being produced, the host cells are also co-transfected with a plasmid comprising a DNA sequence which encodes a heterologous envelope protein, or a plasmid comprising a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration, or both of these plasmids. Alternatively, host cells are transfected with a plasmid comprising a codon optimized DNA sequence encoding a HIV gag protein and a plasmid comprising a codon optimized DNA sequence encoding a HIV pol protein, in place of the plasmid comprising a codon optimized DNA sequence encoding both HIV gagpol proteins.

The present invention also relates to methods of producing viral accessory protein independent lentivirus-derived retroviral vector particles, comprising co-transfecting host cells (e.g., mammalian host cells) with (a) a first plasmid comprising a DNA sequence which encodes lentivirus *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis to improve expression of the lentivirus gagpol proteins; (b) a second plasmid comprising a DNA sequence which encodes a heterologous envelope protein; and (c) a third plasmid comprising a DNA sequence of interest and lentivirus cis-acting sequences required for packaging, reverse transcription and integration. Alternatively, host cells are transfected with a plasmid comprising a codon optimized DNA sequence encoding a lentivirus gag protein and a plasmid comprising a codon optimized DNA sequence encoding a lentivirus pol protein, in place

of the first plasmid comprising a codon optimized DNA sequence encoding both lentivirus gagpol proteins.

In a particular embodiment, the invention relates to methods of producing viral accessory protein independent HIV-derived retroviral vector particles, comprising co-transfecting host cells (e.g., mammalian host cells) with (a) a first plasmid comprising a DNA sequence which encodes HIV *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis to improve expression of the HIV gagpol proteins; (b) a second plasmid comprising a DNA sequence which encodes a heterologous envelope protein; and (c) a third plasmid comprising a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration. Alternatively, host cells are transfected with a plasmid comprising a codon optimized DNA sequence encoding a HIV gag protein and a plasmid comprising a codon optimized DNA sequence encoding a HIV pol protein, in place of the first plasmid comprising a codon optimized DNA sequence encoding both HIV gagpol proteins.

The present invention also relates to viral accessory protein-independent retroviral particles produced by or obtainable by (obtained by) the methods described herein.

The present invention further relates to isolated DNA encoding a codon optimized lentivirus *gagpol*, isolated DNA encoding the *gag* coding region of a codon optimized lentivirus *gagpol*, and isolated DNA encoding the *pol* coding region of a codon optimized lentivirus *gagpol*. In a particular embodiment, the present invention relates to isolated DNA encoding a codon optimized HIV *gagpol*, isolated DNA encoding the *gag* coding region of a codon optimized HIV *gagpol*, and isolated DNA encoding the *pol* coding region of a codon optimized HIV *gagpol*.

The packaging cell lines and viral particles of the present invention can be used for gene therapy or gene replacement with improved safety. The packaging cell lines and viral particles of the present invention can also be used in development and

production of vaccines, and in production of biochemical reagents. Gene therapy vectors produced with the cell lines of the present invention are expected to be valuable medical therapeutics.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of an expression cassette containing the codon optimized *gagpol* genes. The DNA was constructed in multiple segments, which are indicated at the top as 1/3, 2/3, 3/3 (A, B, C and D) and HIN. Restriction sites used to assemble the cloned segments are indicated above the kilobasepair (Kb) ruler. Below the ruler are multiple features showing the location of the human cytomegalovirus (CMV) promoter, human betaglobin sequences (Bglobin), mRNA sequences (thinner line represents intronic sequence), the *gag* and *pol* open reading frames, the individual proteolytic fragment coding sequences (p17_MA, p24_CA, p7, p6, PR, p51_RT, RNaseH and integrase (IN)) and each synthetic oligonucleotide used in the assembly process (multiple adjacent open arrows).

Figure 2 is a table which depicts codon usage frequencies in genes which are highly expressed and in the codon optimized gagpol open reading frame of the HIV packaging construct described herein.

Prior art

B Figure 3 is a schematic representation of the HIV provirus and a three-plasmid expression system used for generating a pseudotyped HIV-based vector by transient transfection as described in Naldini *et al.*, *Science, 272:*263-267 (1996).

Figure 4 is a list of some characteristics relating to the HIV Rev protein.

Figure 5 is a list of some points relating to codon optimization of HIV *gagpol*.

Figure 6 is a partial DNA sequence of HIV *gag* (SEQ ID NO: 1), showing inactivation of inhibitory sequences as described in Schwartz, S. *et al.*, *J. Virol.*, *66*(12):7176-7182 (1992).

Figure 7 a plot of the %(G+C) content of wildtype HIV *gagpol* sequences and theoretically codon optimized HIV *gagpol* sequences. The percent of bases, either G or

C, was calculated for a 30 nucleotide moving window for the entire length of the *gagpol* gene, and the value plotted versus nucleotide position. Diamonds = HIV *gagpol* sequences; squares = full optimal back-translation for *gag* open reading frame; triangles = full optimal back-translation for *pol* open reading frame; CO = codon optimized.

Figures 8A-8E depict the alignment of the nucleotide sequences and predicted amino acid sequences for the *gag* coding region of a wildtype HIV *gagpol* and a codon optimized HIV *gagpol*. "NL4-3 genbank.SEQ" indicates the nucleotide sequence (SEQ ID NO:2) and predicted amino acid sequence (SEQ ID NO:3) for the *gag* coding region of a wildtype HIV *gagpol*. "pHDMHgpm2.seq" indicates the nucleotide sequence (SEQ ID NO:4) and predicted amino acid sequence (SEQ ID NO:5) for the *gag* coding region of a codon optimized HIV *gagpol*. The "NL4-3 genbank.SEQ" sequences are publicly available at the NIH GenBank sequence repository (Accesssion No. M19921).

Figures 9A-9L depict the alignment of the nucleotide sequences and predicted amino acid sequences for the *pol* coding region of a wildtype HIV *gagpol* and a codon optimized HIV *gagpol*. "NL4-3 genbank.SEQ" indicates a nucleotide sequence (SEQ ID NO:6) and a predicted amino acid sequence (SEQ ID NO:7) for the *pol* coding region of a wildtype HIV *gagpol* available in the NIH GenBank sequence repository (Accesssion No. M19921). The nucleotide and amino acid sequences for the *pol* coding region available in the GenBank sequence repository contain two sequence errors, which are indicated in Figures 9A-9L with shading. "pNL4-3.seq" indicates the correct nucleotide sequence (SEQ ID NO:8) and predicted amino acid sequence (SEQ ID NO:9) for the *pol* coding region of a wildtype HIV *gagpol*. "pHDMHgpm2.seq" indicates the nucleotide sequence (SEQ ID NO:10) and predicted amino acid sequence (SEQ ID NO:11) for the *pol* coding region of a codon optimized HIV *gagpol*.

Figures 10A-10D depict the DNA sequence (SEQ ID NO:12) for pHDMHgpm2. The CMV enhancer/promoter is at nucleotides 97 to 679, human betaglobin sequences (Bglobin) are at nucleotides 761 to 864, 865 to 1303 and 5710 to 6469 (end of Bglobin

is at nucleotdes 6445 to 6469), mRNA sequences are at nucleotides 680 to 778 and 1255 to 5921, SV40 origin of replication is at nucleotides 8796 to 8908, beta-lactamase (bla) coding region is at nucleotides 6709 to 7569, intron sequences are at nucleotides 779 to 1254, the codon optimized *gag* coding region is at nucleotides 1318 to 2820, the codon optimized *pol* coding region is at nucleotides 2619 to 5624 and the poly A site is at nucleotides 5897 to 5921.

Figure 11 is a circular map of plasmid pHDMHgpm2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel packaging cell lines useful for generating viral accessory protein independent lentivirus-derived, particularly HIV-derived, retroviral vector particles, to construction of such cell lines and to methods of using the accessory protein independent lentivirus-derived retroviral vector particles to introduce DNA of interest into cells (e.g, eukaryotic cells such as animal (particularly mammalian), plant or yeast cells or prokaryotic cells such as bacterial cells). In a particular embodiment, the packaging cell lines of the present invention are stable packaging cell lines.

The cell lines are engineered to express the lentivirus proteins necessary for virus particle formation (gagpol proteins), without containing DNA sequences from lentivirus accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and Rev response element (RRE)). Additionally, no viral sequences (such as cis-acting elements termed constitutive transport elements (CTEs)) will be expressed as RNA of any kind. DNA sequences for lentivirus *gagpol* are codon optimized by extensively mutagenizing the sequences to improve expression and to reduce the risk of recombination between transfer vector sequences and gagpol messenger RNA. This greatly improves the safety of virus preparations generated from these cell lines. In a particular embodiment, the DNA sequences for lentivirus *gagpol* are not codon optimized in the overlap region

between the *gag* and *pol* sequences and in cis-acting signals necessary for translation of pol.

Examples of lentiviruses include human immunodeficiency viruses (e.g., HIV-1, HIV-2, HIV-3), bovine lentiviruses (e.g., bovine immunodeficiency viruses, bovine immunodeficiency-like viruses, Jembrana disease viruses), equine lentiviruses (e.g., equine infectious anemia viruses), feline lentiviruses (e.g., feline immunodeficiency viruses, panther lentiviruses, puma lentiviruses), ovine/caprine lentiviruses (e.g., Brazilian caprine lentiviruses, caprine arthritis-encephalitis viruses, Maedi-Visna viruses, Maedi-Visna-like viruses, Maedi-Visna-related viruses, ovine lentiviruses, Visna lentiviruses), Simian AIDS retroviruses (e.g., human T-cell lymphotropic virus type 4), simian immunodeficiency viruses, simian-human immunodeficiency viruses, human lymphotrophic viruses (e.g., type III), simian T-cell lymphotrophic viruses.

In another embodiment, cell lines are engineered to express the HIV proteins necessary for virus particle formation (gagpol proteins), without containing DNA sequences from HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and Rev response element (RRE)). Additionally, no viral sequences (such as cis-acting elements termed constitutive transport elements (CTEs)) will be expressed as RNA of any kind. DNA sequences for a HIV *gagpol* are codon optimized by mutagenesis to improve expression and to reduce the risk of recombination between transfer vector sequences and gagpol messenger RNA. In a particular embodiment, the DNA sequences for HIV *gagpol* are not codon optimized in the overlap region between the *gag* and *pol* sequences and in cis-acting signals necessary for translation of pol.

Alternatively, each of the packaging cell lines described herein can be produced using (1) a nucleotide sequence which comprises a codon optimized gag coding sequence and (2) a nucleotide sequence which comprises a codon optimized pol coding sequence, in place of the nucleotide sequence which comprises a codon optimized gagpol coding sequence. In this embodiment, the gag and pol coding sequences can be completely codon optimized

Benefits of the present invention include the removal of potentially harmful lentivirus accessory proteins and other viral sequences, and the reduction of the risk of recombination to produce replication competent virus.

Packaging cell lines for producing a viral accessory protein independent lentivirus-derived retroviral vector particles comprise a mammalian cell and a retroviral nucleotide sequence comprising a coding sequence for a lentivirus *gagpol* which has been codon optimized. In a particular embodiment the packaging cell lines further comprise a retroviral nucleotide sequence comprising a coding sequence for a heterologous envelope protein. In a second embodiment, the packaging cell lines further comprise a retroviral nucleotide sequence comprising a coding sequence for a heterologous envelope protein and a retroviral nucleotide sequence which comprises a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration. In third embodiment, the packaging cell lines further comprise a retroviral nucleotide sequence which comprises a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration. Alternatively, the packaging cell lines of the present invention comprise a retroviral nucleotide sequence which comprises a codon optimized gag coding sequence and (2) a retroviral nucleotide sequence which comprises a codon optimized pol coding sequence, in place of the retroviral nucleotide sequence which comprises a codon optimized gagpol coding sequence.

The coding sequence(s) for lentivirus *gagpol* which has (have) been codon optimized results in improved expression of the lentivirus gagpol proteins and reduces the risk of recombination between the transfer vector and gagpol messenger RNA. Codon optimization of the coding sequence(s) for lentivirus *gagpol* was obtained by mutagenizing for each particular amino acid residue, specific nucleic acid bases in a codon for the particular amino acid residue to a nucleic acid base which is present in a codon which occurs at a high frequency in genes which are highly expressed for the same amino acid residue. In a particular embodiment, the resulting optimized codon

also does not cause introduction of mRNA splicing signals into the codon optimized sequence. Thus, in a particular embodiment, codon optimization of the coding sequence(s) for lentivirus *gagpol* is obtained by mutagenizing for each particular amino acid residue, specific nucleic acid bases in a codon for the particular amino acid residue to a nucleic acid base that is present in a codon which (1) occurs at a high frequency in genes which are highly expressed for the same amino acid residue and (2) does not cause introduction of mRNA splicing signals into the codon optimized sequence. Codon optimization typically results in the removal of nucleic acid base A-rich instability elements.

In a particular embodiment, the coding sequence for a HIV *gagpol* (pNL4-3; available through the AIDS repository, NIH; Adachi *et al., J. Virol.*, 59:284-291 (1986)) has been codon optimized to improve translational efficiency of the HIV gagpol proteins and reduce the risk of recombination between the transfer vector and HIV gagpol messenger RNA. Two hundred thirty-seven base pairs (237 bp) consisting of the gag pol overlap and cis-acting signals necessary for translation of pol (nucleotides 2583 to 2819 of SEQ ID NO: 12) were not optimized. The HIV *gagpol* sequence obtained using the codon optimization process does not differ at the amino acid level from the wildtype HIV *gagpol* sequence, but differs at the nucleotide level from the HIV *gagpol* sequence. A codon optimized HIV *gag* sequence is shown in Figures 8A-8E (pHDMHgpm2.seq) (SEQ ID NO:4). A codon optimized HIV *pol* sequence is shown in Figures 9A-9L (pHDMHgpm2.seq) (SEQ ID NO:10).

A plasmid comprising DNA sequences which encode codon optimized lentivirus *gagpol* proteins is also referred to herein as a packaging construct. This plasmid includes a promoter which drives the expression of the gagpol proteins, such as the human cytomegalovirus (hCMV) immediate early promoter. This plasmid is defective for the production of the viral envelope and accessory proteins tat, vif, vpr, vpu, nef and rev and the Rev response element (RRE). The packaging construct also does not

contain viral sequences which are transcribed into mRNA, such as constitutive transport elements (CTEs).

A packaging construct comprising a codon optimized HIV *gagpol* is depicted in Figure 1 and in Figure 11. Figures 10A-10D depict the DNA sequence (SEQ ID NO:12) for the packaging construct pHDMHgpm2. This packaging construct (pHDMHgpm2) was constructed as follows: Plasmid pMDA.HIVgp mam was generated by chemical synthesis and PCR assembly (which is described in, for example, Stemmer *et al., Gene, 164*:49-53 (1995)) of 215 different oligonucleotides. The DNA sequence for pMDA.HIVgp mam is the same as the DNA sequence for pMDA.HIVgp jtg except for 4.3 kb which was codon optimized using the DNAStar program (LaserGene, Madison, WI). Two hundred thirty-seven base pairs (237 bp) consisting of the gag pol overlap and cis-acting signals necessary for translation of pol (nucleotides 2583 to 2819 of SEQ ID NO: 12) were not optimized due to dual reading frame constraints. A NsiI site 5' of IN was preserved to aid fusion with wildtype sequences. Several single or double base pair silent mutations were introduced either to prevent potential splice donors and acceptors, or by the synthesis process. pMDA.HIVgp jtg was derived from HIV-1 strain NL4-3. The protease mutation that is present in the NL4-3 NIH GenBank sequence was then repaired (Figure 9B), changing the nucleotide present at position 2948 of SEQ ID NO:12 from a "G" to a "C", thereby producing the codon present at nucleotide positions 2948 to 2950 of SEQ ID NO:12 which encodes an arginine instead of the glycine present in the NL4-3 GenBank amino acid sequence. The resulting plasmid was named pMDHgpmam. The EcoRI-HindIII fragment of pMDHgpmam was inserted into pHDM2b, a high copy version of the pMD vector (Ory, D. *et al., Proc. Natl. Acad. Sci. USA,* 93(21):11400-11406 (1996)), to produce plasmid pHDMHgpm. The sequencing mutation that is present in the RNase domain of the NL4-3 NIH GenBank sequence was repaired (Figure 9H), changing the codon present at nucleotide positions 4724 to 4726 of SEQ ID NO:12 from "GGG" to "AAG", thereby producing a codon encoding a lysine instead of the glycine present in

the NL4-3 GenBank amino acid sequence. The resulting plasmid was named pHDMHgpm2. Codon usage frequencies in the codon optimized gagpol open reading frame of the packaging construct pHDMHgpm2 are shown in Figure 2.

As used herein, a heterologous envelope protein permits pseudotyping of particles generated by the packaging construct and includes the G glycoprotein of vesicular stomatitis virus (VSV G) and the amphotropic envelope of the Moloney leukemia virus (MLV). A plasmid comprising a DNA sequence which encodes a heterologous envelope protein is also referred to herein as an envelope coding plasmid.

The terms "mammal" and "mammalian", as used herein, refer to any vertebrate animal, including monotremes, marsupials and placental, that suckle their young and either give birth to living young (eutharian or placental mammals) or are egg-laying (metatharian or nonplacental mammals). Examples of mammalian species include humans and other primates (e.g., monkeys, chimpanzees), rodents (e.g., rats, mice, guinea pigs) and ruminents (e.g., cows, pigs, horses).

Examples of mammalian cells include human (such as HeLa cells, 293T cells, NIH 3T3 cells), bovine, ovine, porcine, murine (such as embryonic stem cells), rabbit and monkey (such as COS1 cells) cells. The cell may be a non-dividing cell (including hepatocytes, myofibers, hematopoietic stem cells, neurons) or a dividing cell. The cell may be an embryonic cell, bone marrow stem cell or other progenitor cell. Where the cell is a somatic cell, the cell can be, for example, an epithelial cell, fibroblast, smooth muscle cell, blood cell (including a hematopoietic cell, red blood cell, T-cell, B-cell, etc.), tumor cell, cardiac muscle cell, macrophage, dendritic cell, neuronal cell (e.g., a glial cell or astrocyte), or pathogen-infected cell (e.g., those infected by bacteria, viruses, virusoids, parasites, or prions).

Typically, cells isolated from a specific tissue (such as epithelium, fibroblast or hematopoietic cells) are categorized as a "cell-type." The cells can be obtained commercially or from a depository or obtained directly from an animal, such as by

biopsy. Alternatively, the cell need not be isolated at all from the animal where, for example, it is desirable to deliver the virus to the animal in gene therapy.

To produce the cell lines of the present invention for producing a viral accessory protein independent lentivirus-derived retroviral vector particles, mammalian host cells are co-transfected with (a) a first plasmid comprising DNA sequence which encode lentivirus *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis, as described above, to improve expression of the lentivirus gagpol proteins; and (2) a second plasmid comprising a DNA sequence which encodes a heterologous envelope protein, or a retroviral nucleotide sequence which comprises a DNA sequence of interest and lentivirus cis-acting sequences required for packaging, reverse transcription and integration, or both, under conditions appropriate for transfection of the cells.

In a particular embodiment, to produce the cell lines of the present invention for producing viral accessory protein independent HIV-derived retroviral vector particles mammalian host cells were cotransfected with (a) a first plasmid comprising DNA sequence which encode HIV *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis, as described above, to improve expression of the HIV gagpol proteins; and (2) a second plasmid comprising a DNA sequence which encodes a heterologous envelope protein, or a retroviral nucleotide sequence which comprises a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration, or both, under conditions appropriate for transfection of the cells.

Virus stocks consisting of viral accessory protein independent lentivirus-derived, particularly HIV-derived, retroviral vector particles of the present invention are produced by maintaining the transfected cells under conditions suitable for virus production (e.g., in an appropriate growth media and for an appropriate period of time). Such conditions, which are not critical to the invention, are generally known in the art. See, e.g., *Sambrook et al., Molecular Cloning: A Laboratory Manual*, Second Edition,

Cold Spring Harbor University Press, New York (1989); *Ausubel et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1998); U.S. Patent No. 5,449,614; and U.S. Patent No. 5,460,959, the teachings of which are incorporated herein by reference.

To generate viral accessory protein independent lentivirus-derived retroviral vector particles, mammalian host cells can be co-transfected with (a) a first plasmid comprising DNA sequence which encode lentivirus *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis, as described above, to improve expression of the lentivirus gagpol proteins; (b) a second plasmid comprising a DNA sequence which encodes a heterologous envelope protein; and (c) a third plasmid comprising a DNA sequence of interest and lentivirus cis-acting sequences required for packaging, reverse transcription and integration. Alternatively, mammalian cells are transfected with a plasmid comprising a codon optimized DNA sequence encoding a lentivirus gag protein and a plasmid comprising a codon optimized DNA sequence encoding a lentivirus pol protein, in place of the first plasmid comprising a codon optimized DNA sequence encoding both lentivirus gagpol proteins. Alternatively, mammalian host cells are transfected with a plasmid comprising a codon optimized DNA sequence encoding a lentivirus gag protein and a plasmid comprising a codon optimized DNA sequence encoding a lentivirus pol protein, in place of the first plasmid comprising a codon optimized DNA sequence encoding both lentivirus gagpol proteins.

In a particular embodiment, the invention relates to methods of producing viral accessory protein independent HIV-derived retroviral vector particles, comprising co-transfecting mammalian host cells with (a) a first plasmid comprising DNA sequence which encode HIV *gagpol* proteins, wherein said DNA sequence has been codon optimized by mutagenisis, as described above, to improve expression of the HIV gagpol proteins; (b) a second plasmid containing a DNA sequence which encodes a heterologous envelope protein; and (c) a third plasmid comprising a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and

integration. Alternatively, mammalian host cells are transfected with a plasmid comprising a codon optimized DNA sequence encoding a HIV gag protein and a plasmid comprising a codon optimized DNA sequence encoding a HIV pol protein, in place of the first plasmid comprising a codon optimized DNA sequence encoding both HIV gagpol proteins.

Virus particles produced by the methods described herein, using a codon optimized HIV packaging construct produced as described herein, were compared by Western analysis with virus particles produced as described in Naldini *et al.*, *Science, 272*:263-267 (1996), using the packaging construct plasmid pCMVΔR8.2. Both the immunological reactivity and the proteolytic processing were confirmed to be indistinguishable.

A plasmid comprising a DNA sequence of interest and HIV cis-acting sequences required for packaging, reverse transcription and integration is also referred to herein as a transfer vector. A transfer vector, as used herein, refers to a vehicle which is used to introduce a DNA of interest into a eurkaryotic cell, particularly a mammalian cell. Figure 3 depicts an example of a transfer vector.

DNA sequence of interest, as used herein, include all or a portion of a gene or genes encoding a nucleic acid product whose expression in a cell or a mammal is desired. In a particular embodiment, the nucleic acid product is a heterologous therapeutic protein. Examples of therapeutic proteins include antigens or immunogens, such as a polyvalent vaccine, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, growth factors, such as epidermal growth factor, human growth factor, insulin-like growth factors, fibroblast growth factors), blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers or "suicide proteins", such as thymidine kinase (including the HSV, CMV, VZV TK), anti-angiogenic factors, Fc receptors, plasminogen activators, such as t-PA, u-PA and streptokinase, dopamine, MHC, tumor suppressor genes such as p53 and Rb,

monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels, such as a calcium channel or a potassium channel, adrenergic receptors, hormones (including growth hormones) and anti-cancer agents. In another embodiment, the nucleic acid product is a gene product to be expressed in a cell or a mammal and which product is otherwise defective or absent in the cell or mammal. For example, the nucleic acid product can be a functional gene(s) which is defective or absent in the cell or mammal.

DNA sequence of interest includes DNA sequences (control sequences) which are necessary to drive the expression of the gene or genes. The control sequences are operably linked to the gene. The term "operably linked", as used herein, is defined to mean that the gene is linked to control sequences in a manner which allows expression of the gene (or the nucleic acid sequence). Generally, operably linked means contiguous.

Control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites and sequences which control termination of transcription and translation. In a particular embodiment, a recombinant gene encoding a desired nucleic acid product can be placed under the regulatory control of a promoter which can be induced or repressed, thereby offering a greater degree of control with respect to the level of the product produced.

As used herein, the term "promoter" refers to a sequence of DNA, usually upstream (5') of the coding region of a structural gene, which controls the expression of the coding region by providing recognition and binding sites for RNA polymerase and other factors which may be required for initiation of transcription. Suitable promoters are well known in the art. Exemplary promoters include the SV40, CMV and human elongation factor (EFI) promoters. Other suitable promoters are readily available in the art (see, e.g., Ausubel *et al., Current Protocols in Molecular Biology,* John Wiley & Sons, Inc., New York (1998); Sambrook *et al., Molecular Cloning: A Laboratory*

Manual, 2nd edition, Cold Spring Harbor University Press, New York (1989); and U.S. Patent No. 5,681,735).

A DNA sequence of interest can be isolated from nature, modified from native sequences or manufactured *de novo*, as described in, for example, Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1998); and Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor University Press, New York. (1989). DNA sequences can be isolated and fused together by methods known in the art, such as exploiting and manufacturing compatible cloning or restriction sites.

The packaging cell lines and viral particles of the present invention can be used, in vitro, in vivo and ex vivo, to introduce DNA of interest into a eukaryotic cell (e.g., a mammalian cell) or a mammal (e.g., a human or other mammal or vertebrate). The cells can be obtained commercially or from a depository or obtained directly from a mammal, such as by biopsy. The cells can be obtained from a mammal to whom they will be returned or from another/different mammal of the same or different species. For example, using the packaging cell lines or viral particles of the present invention, DNA of interest can be introduced into nonhuman cells, such as pig cells, which are then introduced into a human. Alternatively, the cell need not be isolated from the mammal where, for example, it is desirable to deliver vial particles of the present invention to the mammal in gene therapy.

Ex vivo therapy has been described, for example, in Kasid *et al., Proc. Natl. Acad. Sci. USA, 87*:473 (1990); Rosenberg *et al., N. Engl. J. Med., 323*:570 (1990); Williams *et al., Nature, 310*:476 (1984); Dick *et al., Cell, 42*:71 (1985); Keller *et al., Nature, 318*:149 (1985); and Anderson *et al.*, United States Patent No. 5,399,346.

Methods for administering (introducing) viral particles directly to a mammal are generally known to those practiced in the art. For example, modes of administration include parenteral, injection, mucosal, systemic, implant, intraperitoneal, oral, intradermal, transdermal (e.g., in slow release polymers), intramuscular, intravenous

including infusion and/or bolus injection, subcutaneous, topical, epidural, etc. Viral particles of the present invention can, preferably, be administered in a pharmaceutically acceptable carrier, such as saline, sterile water, Ringer's solution, and isotonic sodium chloride solution.

The dosage of a viral particle of the present invention administered to a mammal, including frequency of administration, will vary depending upon a variety of factors, including mode and route of administration; size, age, sex, health, body weight and diet of the recipient mammal; nature and extent of symptoms of the disease or disorder being treated; kind of concurrent treatment, frequency of treatment, and the effect desired.

The teachings of all the articles, patents, patent applications and GenBank sequences cited herein are incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.